Attenuated response of hippocampal AMPK: a neurometabolic account of cognitive aging

Sooah Jang

Department of Medicine

The Graduate School, Yonsei University

Attenuated response of hippocampal AMPK: a neurometabolic account of cognitive aging

Directed by Professor Eosu Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Sooah Jang

This certifies that the Doctoral Dissertation
of Sooah Jang is approved.

Thesis Supervisor : Eosu Kim

Thesis Committee Member#1 : Kee Namkoong

Thesis Committee Member#2 : Chul Hoon Kim

Thesis Committee Member#3: Kang Soo Lee

Thesis Committee Member#4: Ho-Geun Yoon

The Graduate School
Yonsei University

December 2016

ACKNOWLEDGEMENTS

First of all, I greatly and sincerely appreciate my thesis supervisor, Prof. Eosu Kim. During the course of my degree, he provided invaluable support with his warm heart and lively intellectual mind. He always has shown how to live as a good psychiatrist and human being, with a great love of the truth, scientific truth as well as personal truth.

I am also very grateful to Prof. Kee Namkoong, for sustained kind support and inspiration about research and work. Thanks to Prof. Chul Hoon Kim for teaching me about pleasure of basic science. I would also like to express my gratitude to Prof. Kang Soo Lee for time and valuable suggestions which enriched discussion, and to Prof. Ho-Geun Yoon for careful and sincere comments on the thesis. And, I greatly thank to all the professors and colleagues in Department of Psychiatry in Yonsei university college of medicine, for developing me as an independent psychiatrist.

I am also profoundly grateful to all the colleagues in our laboratory, LAMP (Laboratory for Alzheimer's Molecular Psychiatry) for your precious help and the time spent with.

I give my sincere thanks to my family: my father, who always has been a best psychiatrist for me; my mother, to whom my gratitude is beyond expression; my sister, for just being herself; my grandmother, who supported me all the time since I was born, even in the heaven. Lastly, thanks to my husband and daughter, for giving priceless happiness to my life.

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ABSTRACT

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Cognitive aging may be caused by aging-associated impairment in neuronal energy metabolism. AMP-activated protein kinase (AMPK) plays a pivotal role in regulating energy homeostasis, and its adequate activation is crucial to meet metabolic demands. However, it has been found that the sensitivity of AMPK is decreased during aging in responding to an activator or energy stress in peripheral tissues. This may explain bioenergetic failures seen in various organs during aging. So I aimed to see whether the responsiveness of AMPK is also diminished in the aging brain, and thereby associated with cognitive aging. I intraperitoneally administered 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; AMPK activator) or saline (sham) to young (16-week-old) and old (72-week-old) mice and measured daily cognitive performances using the eight-arm maze. AICAR improved overall cognition in young, but not

old, mice, suggesting a blunted effect of AMPK activation on cognition in old mice. AICAR-induced changes in AMPK activity were observed in the hippocampus of young mice after acute intracerebroventricular (i.c.v.) injection. However, neither subchronic nor acute treatment induced significant changes in AMPK activity in old mice. Intriguingly, directions of AICAR-induced changes in AMPK activity were opposite between the hippocampus (decrease) and skeletal muscle (increase). Moreover, hippocampal AMPK activity was inversely correlated with cognitive scores. I hypothesized that higher energy levels successfully achieved by AICAR treatment might be the reason for deactivated neuronal AMPK in young mice. In experiment of acute i.c.v. injection of AICAR, as expected, ATP levels were inversely correlated with hippocampal AMPK activity, suggesting that it was ATP which mediated cognitive improvement by AICAR. Consequences of attenuated response of AMPK were also identified in terms of neurogenesis and brain-derived neurotrophic factor (BDNF) expression, both of which were also increased only in young mice treated with AICAR. The results of my study show that blunted response of neuronal AMPK might be a neurometabolic account of cognitive aging as a composite result from inadequate mobilization in ATP, BDNF, and neurogenesis. These findings suggest that, as well as activating AMPK, recovering its sensitivity may be also required to ameliorate cognitive decline with aging.

Key words: AMPK, Cognitive aging, Neurometabolism, Neurogenesis, BDNF, Hippocampus

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I. INTRODUCTION

1. Cognitive aging

Cognitive aging implies the cognitive state influenced by age itself, which is not only quantitatively, but also qualitatively different from dementia.[1] With an increase of aged population, cognitive decline with aging becomes a highly concerning issue in that it can affect quality of life and health outcomes of the elderly, especially in those whose cognitive decline is below the threshold for the diagnosis of dementia.[2] The prevalence of cognitive impairment without dementia is higher than that of dementia[3]; therefore, developing strategies to slow the cognitive aging process could have a considerable impact on our aging society. Possible contributors for non-pathological cognitive aging such as genetic,

general medical, physiological, dietary and lifestyle has been widely studied.[4] However, unfortunately, neurobiological underpinning of cognitive aging still remains elusive, primarily because a number of factors influence the complex process of brain aging.

A. Cognitive aging and energy metabolism

General aging is characterized by energy deficiency.[5] Likewise, cognitive aging can be explained by dysfunctional energy metabolism in the brain. This hypothesis is supported by neuroimaging studies, which revealed that several regions of the brain show mild hypometabolism during the progression of normal aging.[6,7] An experimental study also shows that energy dysfunction can cause impairment in neurogenesis, synaptic plasticity and following memory impairment that is typical in the aging brain.[8-10] Thus, factors that are critically involved in energy metabolism could be a target of interest in research on cognitive aging.[11]

2. Age-related decreased neurogenesis and metabolism

Adult neurogenesis in the hippocampus has been known as an essential factor of cognitive function.[12] This occurs throughout the life span, however its rate declines with increasing age, which is deeply related with cognitive aging.[13] The aging-associated reduction in adult neurogenesis is partly explained by decline in responsiveness of neural stem cells to various environmental cues such as metabolic stimulation by several growth factors.[13-17] Recently, dysfunctional

energy metabolism has emerged as a key factor for reduced adult neurogenesis.[17] For example, studies have shown that several conditions causing resistance to metabolic signaling, such as high-fat diet, obesity or type 2 diabetes mellitus, lead to impaired neurogenesis,[18-21] while improving energy metabolism by exercise or calorie restriction contributes to enhanced neurogenesis.[22,23] In this regard, aging-related decline in neural responsiveness to metabolic stimuli might be associated with diminished neurogenesis in old age.[13,24]

3. AMPK

A. General structure and function

AMP-activated protein kinase (AMPK) is a pivotal enzyme regulating energy metabolism.[25,26] AMPK is a heterotrimer complex composed of a catalytic alpha subunit with a conventional kinase domain, and two regulatory subunits, beta and gamma.[25] It was first discovered as a protein kinase associated with acetyl-CoA carboxylase and HMG-CoA reductase, which were respectively the rate-limiting regulatory enzymes for fatty acid and cholesterol synthesis.[27,28] After that, this enzyme was known as regulated by “adenylate energy charge”, which implies allosterical modulation by the ratio of [ATP]/[ADP][AMP].[29] In other words, this enzyme senses the ATP:AMP ratio in cells to maintain energy homeostasis. When the ATP:AMP ratio decreases (i.e., there is an energy deficiency), AMPK is activated to enhance cellular catabolism, which results in a net increase in ATP levels.[30] Conversely, when ATP:AMP ratio increases (ATP abundance), AMPK is deactivated, which implies sufficient cellular energy. As expected, altered

functionality of AMPK has been associated with multiple metabolic disorders, such as obesity, type 2 diabetes, and cardiovascular disorders.[31-33]

B. AMPK and cognitive function

AMPK also plays a key role for metabolic control in neurons, which are metabolically highly active, but have low capacity for nutrient storage.[34] In accordance with an importance of AMPK in the brain, several studies have demonstrated a positive effect of AMPK activation on cognitive function. Activation of neuronal AMPK by various stimuli such as drug, natural compound, exercise, dietary restriction or electroacupuncture improved cognitive function in animal studies.[35-39] In a population study, a genetic polymorphism of AMPK is associated with cognitive impairment in the community-dwelling elderly.[40] These results indicated that intact activity of AMPK would be important for maintenance of cognitive function.

C. AMPK and neurogenesis

Consistent with such an involvement in cognitive function, neuronal AMPK is significantly associated with neurogenesis.[34,35] Previous studies have shown that activation of neuronal AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; an AMPK activator) enhances neurogenesis as well as cognitive function in rodents.[35,36,41] Also, neuronal AMPK is among key signaling pathways to expression of brain-derived neurotrophic factor (BDNF), an essential mediator for neurogenesis.[42,43] Such an involvement of AMPK in neurogenesis might mediate relationship between AMPK and cognitive function.

D. Changes of AMPK activity by aging

Interestingly, it has been found that, in peripheral tissues, responsiveness of AMPK to various stimuli is attenuated in old age.[44-46] For example, activation of muscular AMPK in response to AICAR or exercise was blunted in old mice in contrast with its successful activation leading to increased mitochondrial biogenesis in young mice.[46] Consistently, AMPKα2 activity measured upon exercise training was lower in old than young individuals.[44]

4. Aims of this study

Discovering neurobiological underpinning of cognitive aging would be an essential research in aging society. Recently, metabolic failure of brain has known to be largely responsible for cognitive aging, as general aging is mainly caused by metabolic dysfunction.

AMPK is a pivotal enzyme for metabolic homeostasis of cell as well as organism. Moreover, neuronal AMPK activity plays an active part in maintenance of cognitive function and neurogenesis. Such an AMPK activity has found to be blunted in old age, and this attenuated AMPK activity has influenced on function of the organ, which were identified in peripheral tissues. However, to my knowledge, few experimental studies have shown directly whether neuronal AMPK action is indeed attenuated during aging and such attenuation is related to cognitive aging.

Therefore, I aimed to examine the effect of aging on sensitivity of neuronal AMPK in responding to its activator, and the association of AMPK activity with cognition as well as with other cognitive effectors such as BDNF and neurogenesis in young versus old mice

II. MATERIALS AND METHODS

1. Animals and drug treatment

Young (16-week-old) and old (72-week-old) male C57BL/6 mice (Animal Facility of Aging Science, Kwangju, Republic of Korea) were used in this study and housed under a 12-hr light/dark cycle with food and water given ad libitum. All procedures for animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University Health System and performed according to the National Institute of Health guidelines for the Care and Use of Laboratory Animals.

AICAR (sc-200659, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in saline. For subchronic experiments, mice were injected intraperitoneally (i.p.) with a dose of 125 mg/kg/day AICAR or saline vehicle, for 7 days. Injections were performed from 10:00 am to 12:00 pm and behavior tests were performed 3 hr after injection. Mice were sacrificed on the 7th day, about 5 hr after last drug treatment (Fig. 1). The number of mice in the first subchronic experiment was 6 per group, and additional 4 mice per group were used in the second experiment for analysis of muscle. For the results of neuronal tissues, data from a total of 10 mice per group were pooled to be analyzed since the direction of changes in neuronal AMPK activity upon AICAR treatment in young mice were the same in both the first (n = 6 per group) and second (n = 4 per group) cohorts. For acute experiments, AICAR (25 nM) or saline was administered intracerebroventricularly (i.c.v.) at a rate of 0.2 μL/min by

stereotaxic injection using a Hamilton syringe at the coordinates of 0.2 mm posterior, 1.0 mm lateral, and 3.1 mm deep from bregma. Surgery was performed under isoflurane anesthesia (2.0% isoflurane in 30% oxygen and 70% nitrous oxide using a facemask). Brains were extracted about 3 hr after i.c.v treatment. The number of mice in acute experiment was 3 per group.

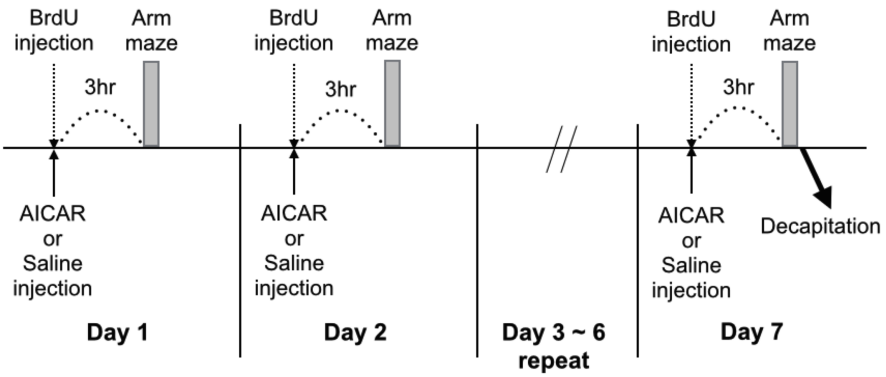

Figure 1. Schematic representation of the behavioral test and drug treatment. Mice were treated with vehicle (control) or 125 mg/kg/day AICAR intraperitoneally for 7 days, and tested in the eight-arm maze 3 hr after the injection every day.

2. Behavioral test: the eight-arm maze

The eight-arm maze tests and data processing were performed as described previously.[35] The maze was slightly modified size of version for mice,[47] which consisted of eight arms (32 cm long × 4cm wide × 4cm high) extending radially from an octagonal central platform (18 cm in diameter). The apparatus was transparent and surrounded by various and consistent extramaze cues such as a chair, bottles and laboratory benches. Mice were water-deprived overnight, and 50 μl of water was presented as a reward at the end of each arm. Mice were placed in the maze 3 hr after drug treatment for 7 consecutive days (Fig. 1). The test ended when the mice: entered into all 8 arms, or entered any arm 24 times whichever comes first. Overall performance score during the whole test period was determined by the area under the curve (AUC) analysis utilizing the formula: 6 × (average number of entries of each group in day 1) − (number of entries of each mouse in day 2 + day 3 + day 4 + day 5 + day 6 + day 7).

3. Western blot analysis

For western blot analysis, hippocampi and right anterior quadriceps muscle were immediately dissected and prepared after sacrifice of animals. Hippocampal and muscle tissues were homogenized in lysis buffer consisting of: 20 mM Hepes (pH 7.0), 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, and cocktails of phosphatase inhibitors and protease inhibitors. Homogenates were centrifuged at

8,000 × g for 30 min. Equal amounts of each protein sample were resolved on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking, membranes were probed overnight at 4°C with the following antibodies: anti-Thr^{172}-phosphorylated AMPKα (p-AMPK; 1:1,000, 2531s, Cell Signaling Technology, Beverly, MA, USA), anti-AMPKα (1:1,000, 2603s, Cell Signaling Technology). Membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000, 7074s, Cell Signaling Technology) for 1 hr at room temperature (RT), followed by enhanced chemiluminescence detection (ECL plus; Amersham Biosciences, Piscataway, NJ, USA). Measurement of signal intensity was done by Multi Gauge 3.0 analysis software (FUJIFILM, Tokyo, Japan). Signal intensity of p-AMPK was normalized by that of total AMPK, and the absolute value of p-AMPK/AMPK ratio was converted to relative values by the reference value (p-AMPK/AMPK set as 1 from a randomly selected, young control mouse).

4. ATP assay

ATP assays were performed once with hippocampi, which were dissected 3 hr after i.c.v. drug treatment. Fresh hippocampal tissues were homogenized with sterilized ice-cold phosphate-buffered saline (PBS). Homogenates were centrifuged for 30 sec and supernatants were transferred into new tubes. The supernatant (10 μl) was analyzed for ATP levels using the colorimetric ATP assay kit (BioVision, Mountain View, CA, USA). The remaining supernatant was quantified for protein analysis. Concentration of ATP was presented as amount of

ATP per mg of protein.

5. Assessment of neurogenesis

For analysis of neurogenesis in vivo, 5-bromo-2-deoxyuridine (BrdU; B5002, Sigma-Aldrich Co., St. Louis, MO, USA) was injected i.p. at a dose of 100 mg/kg/day for 7 consecutive days. Mice were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA). The brains were removed and post-fixed overnight in 4% PFA followed by equilibration in 15% and 30% sucrose for 24 hr each. Tissues were then quick-frozen in optimal cutting temperature compound (Sakura Finetek, Zoeterwoude, the Netherlands) and cut coronally into 30 μm sections through the anteroposterior extension of the hippocampi using a cryostat. Every sixth section from each brain was mounted onto gelatin-coated slides and processed for immunohistochemistry. I pretreated sections for permeabilization (with 0.3% Triton-X-100 in PBS at RT for 1 hr), and quenching (with 3% H2O2 in methanol at RT for 40 min). For BrdU staining, I performed DNA denaturation (with 2× saline-sodium citrate/50% formamide at 65°C for 2 hr and 2N HCl at 37°C for 30 min, followed by a rinse in 100 mM sodium borate pH 8.5) on pretreated-sections. Sections were then washed with PBS and blocked for 1 hr with Mouse on Mouse (MOM) blocking solution (MOM kit, BMK-2202, Vector Laboratories, Burlingame, CA, USA). Afterward, sections were again washed with PBS, incubated for 5 min with diluent solution (MOM kit) and then incubated for 1.5 hr with a mouse anti-BrdU antibody (1:200, B8434, Sigma-Aldrich Co.). Next, sections were again washed with PBS and incubated for 30

min with the secondary antibody biotinylated anti-mouse Ig (1:250, MOM kit, Vector Laboratories). For doublecortin (DCX) staining, pretreated-sections were blocked for 1 hr with 3% bovine serum albumin (BSA; Sigma-Aldrich Co.), followed by incubating with a goat anti-DCX antibody (1:200, sc-8066, Santa Cruz Biotechnology) overnight. Then, sections were washed with PBS and incubated for 1 hr with a biotinylated anti-goat IgG (1:250, BA-9500, Vector Laboratories). For both immunostaining, immunoreactive sites were visualized using the Vectastain ABC kit (Vector Laboratories) and the Ni-DAB (Vector Laboratories) method. Counterstaining was done with nuclear fast red (Vector Laboratories). After immunostaining, the cryostat sections were imaged with a light microscope (Olympus, Pennsylvania, PA, USA). The cells that were positive for BrdU or DCX were counted in the dentate gyrus (DG; including the granular and subgranular zone) in the 40 x magnified images. DCX positive cells were counted only if they had extended neural process at least to a minimal degree. Counting was performed by who was blind to treatment and age conditions. Total number of positive cells in the whole hippocampus of each animal was calculated by multiplying 6 by the number of positive cells summed up from every sixth section from a single hippocampus.

6. BDNF immunohistochemistry

Fixed coronal hippocampal sections were permeabilized in PBS with 0.3% Triton-X-100 and stained in free-floating conditions. After blocking, sections were incubated with the primary antibody goat anti-BDNF (1:200, sc-546, Santa

Cruz Biotechnology) in PBS containing 2% bovine serum albumin and 0.3% Triton-X-100 at 4°C overnight. After washing with PBS three times, slices were labeled with the secondary antibody donkey anti-goat Alexa Fluor® 488 (705-546-147, Jackson Immuno Research Laboratories, West Grove, PA). Cells were counter-stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and observed under a confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). The fluorescence intensity was quantified in a 20 x magnified field containing each hippocampal subfield (CA1, CA3 and DG) using Zen 2012 imaging software (Carl Zeiss MicroImaging GmbH, Germany). Measurement was performed by an experimenter blind to age or treatment condition. Values were corrected for background intensity in the selected field area for each section.

7. Statistical analysis

I conducted 2x2 factorial Analysis of Variance (ANOVA) with factors as age (young versus old) and treatment (saline versus AICAR). For post hoc comparison between saline and AICAR treatment within each group of the same age, t-test were used with unadjusted p-value. Correlations were evaluated by Pearson's correlation analysis. Bar graphs indicate mean ± standard error (SE). Statistical significance was set at two-tailed $p < 0.05$. All analyses were performed using SPSS 20 (IBM Corp., Armonk, NY, USA).

III. RESULTS

1. Improvement in cognition upon AICAR treatment was attenuated in old versus young mice

I first examined the effect of AICAR treatment on cognitive function in old versus young mice using the eight-arm maze.35 For 7 days, mice were daily i.p. injected with a dose of 125 mg/kg/day AICAR or saline and performed the maze test 3 hr after the injection (Fig. 1). Evaluating overall effect of treatment on cognitive performance throughout the 7 days using area AUC analysis, I found the main effect of treatment [$F(1, 28) = 5.769$, $p = 0.023$] and of age x treatment interaction [$F(1, 28) = 6.270$, $p = 0.018$] on cognitive improvement in 2-way ANOVA. Age itself had no significant effect [$F(1, 28) = 1.632$, $p = 0.212$]. AICAR treatment had a significant effect on cognition in young mice ($t = -3.213$, $p = 0.006$; Fig. 2a) but not in old mice ($t = 0.079$, $p = 0.938$; Fig. 2a).

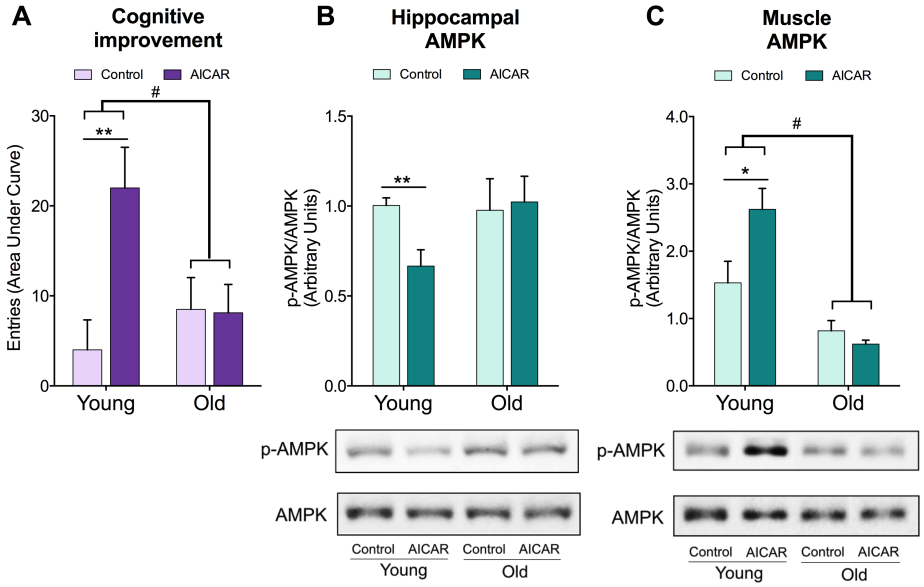

Figure 2. Cognitive improvement and response of hippocampal and muscle AMPK upon subchronic AICAR treatment in old versus young mice.

Mice were given vehicle (control) or AICAR (125 mg/kg/day) intraperitoneally for 7 days. Cognitive improvement was tested in the eight-arm maze. (A) The overall performance on the eight-arm maze was formulated as AUC (n = 8/group). A higher AUC value indicates better performance. AMPK expression and phosphorylation on Thr^{172} were analyzed by Western blot on the final day of drug administration. (B) Entire hippocampus (n = 10/group). (C) Right anterior quadriceps muscle (n = 4/group). AUC, Area under the curve. Main effect of age, treatment, and age x treatment interaction was analyzed by 2-way ANOVA. # $p < 0.05$ for interaction. The effect of treatment within each age-group by t-test (unadjusted). * $p < 0.05$, ** $p < 0.01$. Each bar, mean ± SE.

2. Response of hippocampal and muscle AMPK upon AICAR treatment attenuated in old versus young mice

I hypothesized that the age-associated difference in cognition with AICAR treatment could be attributed to attenuated AMPK activation in older brains. Therefore, I measured changes in hippocampal AMPK activity upon AICAR treatment in young and old mice by assessing the ratio of p-AMPK to total AMPK. In 2-way ANOVA, I found no statistically significant main effect of age [F (1, 36) = 1.814, p = 0.186] or of treatment [F (1, 36) = 1.368, p = 0.25] on the levels of hippocampal AMPK activity. Age x treatment interaction was not significant [F (1, 36) = 2.421, p = 0.128; Fig.1a]. However, as can be seen in Fig. 2b, the direction of change in p-AMPK levels following AICAR treatment seemed to be directly opposite to my expectation in young mice (for reference, t = 3.371, p = 0.003, unadjusted for potentially multiple comparisons; Fig. 2b). Therefore, I proceeded to an additional experiment with the small number of animals (n = 4 per group) to see the changes in AMPK activity by AICAR in the skeletal muscles as well. This was to confirm whether 7-days i.p. AICAR treatment had been pharmacologically valid and provided results consistent with previous reports using the skeletal muscles or liver tissues.[45,46] In the levels of muscle AMPK activity, significant main effect of age [F (1, 12) = 32.666, p < 0.001] and age x treatment interaction [F (1, 12) = 7.417, p = 0.018; Fig. 2c] were observed. Treatment [F (1, 12) = 3.548, p = 0.084] showed no significant main effect. In addition, the direction of changes in AMPK activity upon AICAR treatment was ‘increase’ in young mice (t = -2.455, p = 0.049; Fig.2c), which was different from

the findings of the hippocampus. Highly consistent with the previous report,[46] this finding confirmed that AICAR administration was pharmacologically valid in experimental animals of this study.

3. Cognitive function inversely correlated with hippocampal AMPK activity

To address the contextual meaning of the reduced activity of hippocampal AMPK in young mice after AICAR treatment, I examined the relationship between AMPK activity and cognitive scores. Interestingly, I found a significant inverse correlation between cognitive function and hippocampal p-AMPK levels in young mice (r = -0.599, p = 0.040; Fig. 3a), although this correlation was not observed in old mice (r = -0.077, p = 0.812; Fig. 3a) or entire mice (r = -0.305, p = 0.148; Fig. 3a). Muscle AMPK activity and cognitive function showed: significant positive correlation in entire mice (r = 0.600, p = 0.014; Fig. 3b), a trend level in young mice (r = 0.665, p = 0.072; Fig. 3b), and no correlation in old mice (r = 0.249, p = 0.553; Fig. 3b). These results further support the hypothesis that cognitive improvement in young mice is associated with reduced activity of neuronal AMPK upon AICAR treatment, although there is likelihood of involvement of muscle AMPK as well.

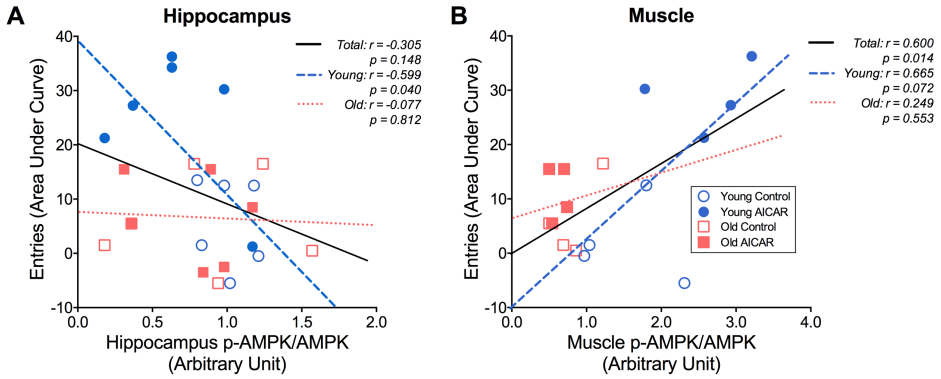

Figure 3. Correlation between cognitive function and AMPK activity of hippocampus and muscle. Seven days after drug administration, relative hippocampal p-AMPK levels and AUC analysis of performance on the eight-arm maze showed: a positive correlation in young mice, but no correlation in the old and entire mice. In the muscle, relative p-AMPK levels and AUC score showed: a positive correlation in entire mice, a trend level in young mice, and no correlation in the old mice. Correlation by Pearson's correlation test. Lines and r values indicate the regression of data (black solid, entire mice; blue dashed, young mice; red dotted, old mice). AUC, Area under the curve.

4. ATP levels inversely correlated with hippocampal AMPK activity

Based on the unexpected finding of the decreased hippocampal p-AMPK level and inverse relationship between hippocampal AMPK activity and cognitive function in young mice upon AICAR treatment, I speculated that a reduction in neuronal AMPK activity might indicate a higher energy state induced by AICAR treatment. That is, if AICAR improved cognition in young mice by successfully promoting ATP production through AMPK signaling, then AMPK activity per se might be deactivated by the increase in ATP levels. To test this hypothesis, I examined the relationship between ATP levels and AMPK activity upon acute AICAR treatment. In this experiment, I used i.c.v. AICAR injection instead of i.p. injection to minimize the secondary effects of AICAR-induced changes in peripheral metabolism on hippocampal AMPK. I found a strong inverse relationship between hippocampal AMPK activity and ATP levels (r = -0.719, p = 0.008; Fig. 4a) regardless of age or treatment. In regard to each age group, there was a trend level of inverse correlation in young mice (r = -0.793, p = 0.060; Fig. 4a), but no correlation was shown in old mice (r = -0.552, p = 0.256; Fig. 4a).

When it comes to the AMPK activity, acute i.c.v. AICAR injection showed significant age x treatment interaction [F (1, 8) = 8.283, p = 0.021; Fig. 3b. Also see Supplementary Table 1], which indicates blunted AMPK response in old mice. The direction of hippocampal AMPK activity in young mice was also ‘decreased’ in accordance with the result from the 7-day systemic (i.p.) treatment.

Regarding the changes in ATP levels, although it seemed that AICAR increased

the mean value only in young mice (Fig. 4c), no significant interaction between age and treatment [F (1, 8) = 1.415, p = 0.268; Fig. 4c. Also see Supplementary Table 1].

Although still elusive to draw a conclusion from this additional experiment with the small number of samples, results from the correlation analyses could suggest that ATP-AMPK feedback loop might indeed exist, and, if so, it would be only apparent in young mice. At least, it was revealed here that response of AMPK was blunted in old mice compared to young mice upon direct AICAR injection into the brain.

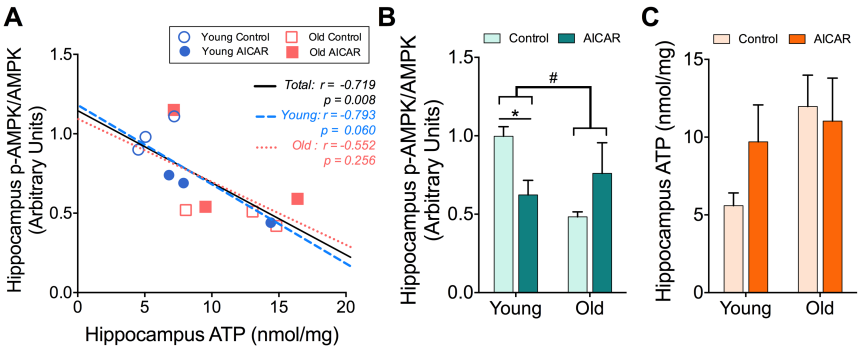

Figure 4. ATP levels inversely correlated with hippocampal AMPK activity. Mice were treated intracerebroventricularly with vehicle (control) or 25 nM AICAR injection and decapitated 2.5 hr after drug administration. In the hippocampus: (A) correlations between ATP level and relative p-AMPK level were analyzed by Western blot analysis, (B) AMPK expression and phosphorylation on Thr^{172} were analyzed by Western blot analysis (n = 3/group), and (C) ATP levels (nmol/mg protein) were analyzed using a colorimetric ATP assay kit (n = 3/group). Correlation by Pearson's correlation test. Line and r value indicate the regression of the dataset (black solid, entire mice; blue dashed, young mice; red dotted, old mice). Main effect of age x treatment interaction by 2-way ANOVA. # $p < 0.05$ for interaction. The effect of treatment within each age-group by t-test (unadjusted). * $p < 0.05$. Each bar, mean ± SE.

5. Neurogenesis upon AICAR treatment attenuated in old versus young mice

Next, I explored the changes in potential mediators between AMPK and cognition, as downstream effectors of AMPK. Based on a previous report showing that AICAR enhances cognitive function via AMPK-dependent increases in hippocampal neurogenesis,35 I compared the levels of AICAR-induced neurogenesis in young versus old mice. For 7 days, mice were daily i.p. injected with a dose of 125mg/kg/day AICAR and 100mg/kg/day BrdU. After 7 days of drug treatment, 2-way ANOVA of number of BrdU- and DCX-immunoreactive cells in the hippocampus revealed a significant main effect of age [BrdU, $F(1, 11) = 487.2$, $p < 0.001$; DCX, $F(1, 11) = 711.407$, $p < 0.001$] and a significant age x treatment interaction [BrdU, $F(1, 11) = 7.564$, $p = 0.019$; DCX, $F(1, 11) = 5.504$, $p = 0.046$; Fig. 5. Also see Supplementary Table 1]. These results suggest that AMPK-dependent increases in neurogenesis might be associated with cognitive improvement, as I observed both results only in young mice (Fig. 2a and Fig. 5).

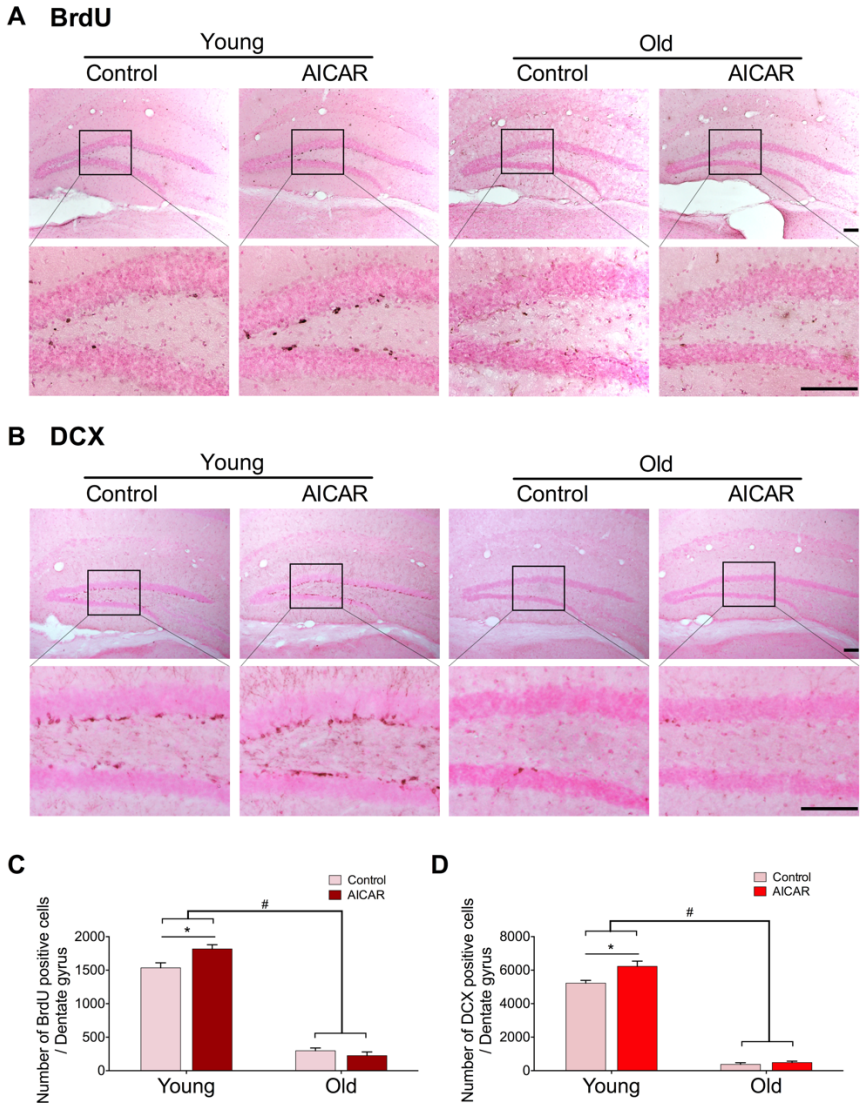

Figure 5. Enhanced neurogenesis upon AICAR treatment was attenuated in old versus young mice. Representative photomicrographs of BrdU (A) and DCX (B) positive cells in the hippocampus after 7-day BrdU (100 mg/kg/day) and AICAR (125 mg/kg/day) injections. Top line, 10×. Bottom line, 40×. Scale bar indicates 100 μm. Number of BrdU (C) and DCX (D) positive cells per

dentate gyrus (n = 3–4/group). BrdU, 5-bromo-2'-deoxyuridine; DCX, doublecortin. Main effect of age (BrdU, $p < 0.001$; DCX, $p < 0.001$) and treatment (BrdU, $p = 0.126$; DCX, $p = 0.017$), and age x treatment interaction (BrdU, $p = 0.019$; DCX, $p = 0.046$) by 2-way ANOVA. # $p < 0.05$ for interaction. The effect of treatment within each age-group by t-test (unadjusted). * $p < 0.05$. Each bar, mean ± SE.

6. BDNF expression with AICAR treatment attenuated in old versus young mice

BDNF is a crucial signal for neurogenesis and is upregulated by AMPK[42,48]; therefore, I investigated whether the effect of AICAR treatment on hippocampal BDNF expression also differed between young and old mice. In the CA3 region of hippocampi, significant main effect of age and treatment [age, F (1, 19) = 12.345, $p = 0.002$; treatment, F (1, 19) = 13.526, $p = 0.002$; Fig. 6a and 6c] were shown on the level of BDNF immunoreactivity, with significant age x treatment interaction [F (1, 19) = 11.726, $p = 0.003$; Fig. 6a and 6c]. These results suggest that AICAR treatment significantly increased BDNF expression in CA3 only from young mice, but not old mice. However, such age x treatment interaction was not observed in other hippocampal regions [CA1, F (1, 20) = 0.141, $p = 0.711$; DG, F (1, 19) = 3.261, $p = 0.087$; Fig 6a, 6b and 6d], with only significant main effect of age in DG [F (1,19) = 6.993, $p = 0.016$, see Supplementary Table 1].

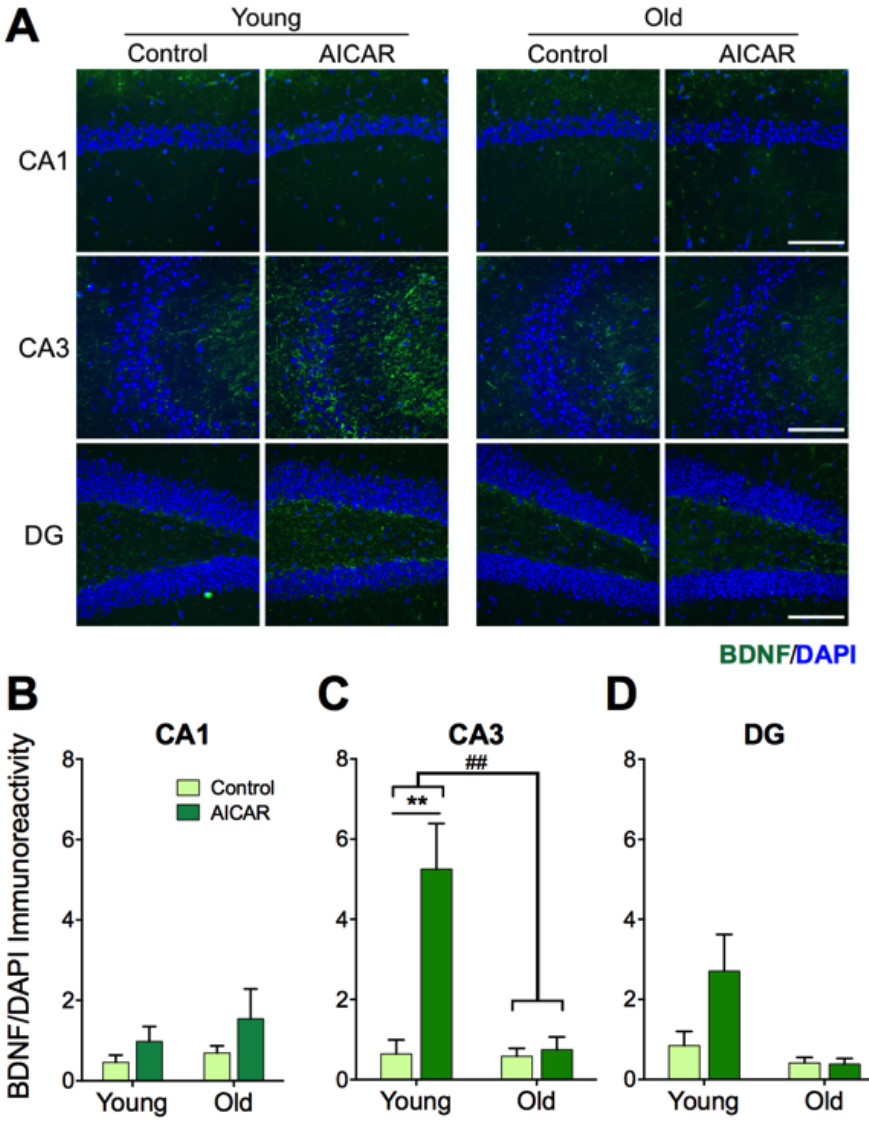

Figure 6. Enhanced BDNF expression upon AICAR treatment was attenuated in old versus young mice. (A) Representative confocal images of hippocampal sections incubated with a BDNF antibody (Blue, DAPI; Green, BDNF). Scale bar indicates 100 μm. Measured immunoreactivity of BDNF in the CA1 (B), CA3 (C), and DG (D) after daily intraperitoneal AICAR (125

mg/kg) injection for 7 consecutive days (n = 3–4/group). BDNF, Brain-derived neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; CA, cornus ammonis; DG, dentate gyrus. Main effect of age (CA1, $p = 0.37$; CA3, $p = 0.002$; DG, $p = 0.016$) and treatment (CA1, $p = 0.129$; CA3, $p = 0.002$; DG, $p = 0.096$), and age x treatment interaction (CA1, $p = 0.711$; CA3, $p = 0.003$; DG, $p = 0.087$) by 2-way ANOVA. ## $p < 0.01$ for interaction.

IV. DISCUSSION

In this study, I tested my hypothesis that reduced activation of neuronal AMPK in old age could cause cognitive aging. Using AICAR as an AMPK activator, I found that significant responses in AICAR-induced AMPK activity occurred in the young, but not in old, mice upon acute i.c.v. AICAR treatment, although this age-related difference was not prominent in subchronic treatment. The effects of subchronic AICAR treatment were still found diminished on cognitive function, neurogenesis, and BDNF expression in old mice compared to young mice. These results suggest that cognitive aging may be considered a form of neuronal bioenergetic failure that could become overt with increased cognitive and metabolic demands.

A reduced response of AMPK and AMPK-related signals upon AICAR treatment has been previously identified in the muscle and liver tissues of old mice.[45,46] In these studies, the authors suggest that the reduction in AMPK activity could be a causative factor for aging-associated metabolic dysfunction.[45,46] In the current study, I found evidence that attenuated AMPK responses in old age also may account for aging-associated neuronal dysfunction (Fig. 7). In this regard, my study suggests that not only developing novel AMPK activators but also enhancing the sensitivity of AMPK could be a key strategy to alleviate age-related reduction in neurogenesis and subsequent cognitive decline.

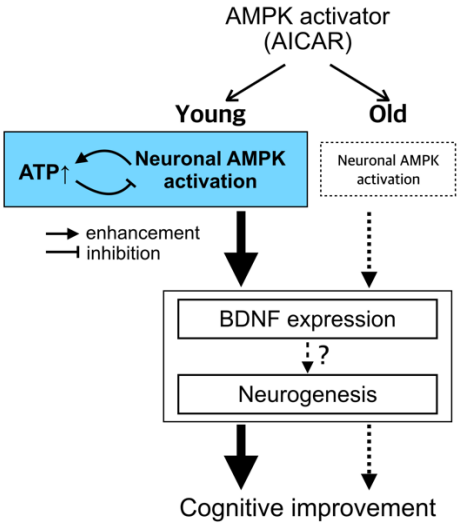

Figure 7. Working hypothesis of the attenuated response of neuronal AMPK and cognitive aging. Schematic representation of the sensitivity of neuronal AMPK and cognitive aging. In young mice, AICAR-induced neuronal AMPK activation successfully increased the level of ATP, which in turn reduced AMPK activity. In old mice, the response of neuronal AMPK and other related pathways attenuated, which may account for cognitive aging. In terms of the BDNF expression and neurogenesis, both of them were not meaningfully increased by AICAR in old age. Although these results could together indicate that the blunted AMPK response (reduced BDNF expression) may be responsible for reduced neurogenesis in old age, region-specific, direct relationship between BDNF and neurogenesis was not supported by our findings that showed significant increases in each component occurred in different regions (CA3 and DG, respectively) in young mice (see the text).

Surprisingly, the direction of the AICAR-induced change in AMPK activity was different between the hippocampal and skeletal muscle tissues in young mice. Upon AICAR treatment, p-AMPK levels were increased in muscle, but seemingly reduced in the hippocampus. Such a reduction in neuronal AMPK activity could be interpreted as a rapid deactivation rather than a direct inhibition, by AICAR. The inverse relationship between ATP levels and hippocampal AMPK activity observed after i.c.v. AICAR injection supports this speculation. Increased ATP levels may be caused by an AICAR-induced increase in oxidative phosphorylation via the mitochondria, because AMPK stimulates mitochondrial biogenesis and ATP production.[49] In addition, given the previous report that deactivation of AMPK occurred in approximately 40 min following AICAR treatment in vitro,[50] 5 hr after i.p. injection or 3 hr after i.c.v. injection in my experiment might be long enough for the feedback mechanism to be apparently working.

An explanation for the difference between AMPK activity in neuronal and muscle tissues is beyond the scope of this study; however, I speculate that this difference might stem from the unique characteristics of neuronal tissue. Neuronal tissues may be vulnerable to prolonged activation or over-activation of AMPK, compared with other tissues.[51-53] Therefore, they may have an innate defense mechanism to avoid unwanted off-target adverse effects beyond meeting [54]metabolic demands.

Another important question that I have had was how senescence reduces

response of AMPK. In this study, we could not certainly identify whether the function of downstream pathways of AMPK changed by aging, but we showed reduced sensitivity of AMPK itself in brain as well as muscles. Although definite reason of its reduced sensitivity has not been found yet, several reports have provided clues about possible underlying mechanisms of aging-associated reduction of AMPK response. Aging-related functional changes of protein phosphatases such as PP2A, PP2Cα or PPm1E, which are involved in the suppression of AMPK activation,[55,56,57] could be one cause of reduced responsiveness. In addition, given that anti-inflammatory agent did a role of potent AMPK activator,[58,59] chronic inflammatory environment in aged tissue might incapacitate sensitivity of AMPK. Another possible mechanism shown by Shao et al.(2014)[60] is that AMPK transforms into oxidized aggregates because of intermolecular disulfide bonds formed under oxidative stress, which negatively regulates AMPK activity. Further studies which verify the mechanism of age-related attenuation of sensitivity of AMPK are needed to identify the connection of neurometabolism and cognitive aging.

This study has several limitations. First, I only used AICAR to modulate AMPK activity. An extended study using AMPK inhibition such as a functional knock-out system would be crucial to show a direct causal relationship between AMPK activity and neurogenesis or BDNF expression, although several previous studies have been supporting such relationships.[36,61,62] On the other hand, given the blunted response to AICAR in old age, a study using any measure enabling recovery of AMPK responsiveness would successfully prove the importance of

AMPK involvement in cognitive aging.

Second, I only showed the results from a relatively short period of AICAR treatment. A recent study shows that prolonged AICAR treatment can induce expressions of genes related to neural development and increase cognitive function in both old and young mice. However, this previous study also suggested the concept of 'reduction in AMPK sensitivity in old mice', since it showed that a longer period of AICAR treatment was needed to improve cognition in old than young mice.[54] Thus, the current study could be evaluated as a direct measurement of hippocampal AMPK activity in order to account for the differential effects of an AMPK activator on neurogenesis between young and old mice within a limited period of treatment. Future studies are needed to identify the molecular mechanisms responsible for the reduced sensitivity of AMPK in old age.

Third, to further address the unexpected finding, I explored the relationship between AMPK activity and ATP levels in the hippocampus following directly injecting AICAR into the ventricles. Although I indeed found the inverse correlation between them, more comprehensive interpretation of data from this acute experiment should be cautious due to the small number of animals used. For example, following this acute injection, AMPK activity in old age was shown comparably as high as in young age, which is elusive to draw logical conclusion. Additionally, since I determined to measure ATP levels to see the potential feedback loop between ATP and AMPK by acute i.c.v. injection of AICAR, I could not further address the issue with the data from muscle tissue, which would

be able to broaden our understanding of the timing and dynamics in the feedback loop across different kinds of tissues.

Fourth, originally, I intended to examine the changes in BDNF levels upon AICAR treatment under a hypothesis that BDNF is a potential candidate that may mediate the link between reduced AMPK action and neurogenesis in old age since BDNF is induced by AMPK activation and enhances neurogenesis. However, besides the methodological limitations in using immunohistochemistry for quantification of protein expressions, my results failed to support the region-specific link between reduced BDNF and neurogenesis in old age. The significant changes in each component were identified in different subregions in the hippocampus; significant changes in neurogenesis were identified in DG while changes in BDNF levels only significant in CA3 region upon AICAR treatment. Therefore, these findings suggest that CA3-BDNF expression may be an independent factor from the changes in DG-neurogenesis upon AICAR treatment. However, the finding of blunted change in BDNF expression in old age may still support the age-related reduction in responsiveness of AMPK as BDNF expression is stimulated by AMPK. In addition, several previous studies have suggested the possible link between BDNF expression in CA3 and neurogenesis in DG enhanced by exercise, diet restriction, or antidepressants.[22,63,64] Thus further studies are warranted to elucidate the region-specific relationship between AMPK, BDNF, and neurogenesis regarding aging process.

Fifth, my study could not determine whether AMPK activation in the brain was

mediated directly by AICAR or secondary to peripheral effects of AICAR[54] for I did not measure brain concentration of AICAR following i.p. injection. A positive correlation between muscle p-AMPK and cognitive function (Fig. 3b) could also raise a question about peripheral effects. However, although the permeability of the blood brain barrier by AICAR has been known to be relatively low,[65] there have been several studies which demonstrated activation of brain AMPK by systemic AICAR administration.[35,66] In addition, not only systemic injection[35,54] but also direct i.c.v. injection of AICAR[67] was verified to improve cognitive function, showing the involvement of AMPK in cognition.

Lastly, I have not investigated the fundamental reason of aging-associated attenuation of AMPK. Further studies, which traces the underlying molecular mechanisms of blunted responsiveness of AMPK, could have an implication on the research of aging-associated metabolic dysfunction.

V. CONCLUSION

This study showed that an aging-associated reduction in hippocampal AMPK sensitivity might be an underlying molecular account of cognitive aging, which is paralleled by a decrease in neurogenesis and BDNF expression in old mice. Blunted AMPK response in old age is a new finding regarding brain tissue, corroborating previous findings in peripheral tissues.[45,46] What I have found through this study is,

1. AICAR improves cognitive function only in young mice, not in old mice.

2. AICAR-induced change in hippocampal AMPK activity was blunted in older mice.

3. AICAR increased neurogenesis and BDNF expression in young, but not in old mice.

4. Blunted AMPK activation may account for age-related reduction in neurogenesis and subsequent cognitive decline.

5. Recovering AMPK responsiveness may be key to enhance cognitive aging.

These results imply that the activation of neuronal AMPK to meet cognitive and neurometabolic demands may be diminished in old age. Thus, further studies should focus on a strategy to recover the sensitivity of AMPK in old age and determine the therapeutic potential of AMPK activation in preventing or alleviating cognitive decline with aging.

Supplementary table 1. Statistics of results by 2x2 factorial ANOVA with factors as age and treatment.

	Variable	Factor	F	df	p
Figure 2a	Entries (AUC)	Age	1.632	1, 28	0.212
		Treatment*	**5.769**	**1, 28**	**0.023**
		Age x Treatment*	**6.270**	**1, 28**	**0.018**
Figure 2b	Hippocampal p-AMPK/AMPK	Age	1.814	1, 36	0.186
		Treatment	1.368	1, 36	0.25
		Age x Treatment	2.421	1, 36	0.128
Figure 2c	Muscle p-AMPK/AMPK	**Age***	**32.666**	**1, 12**	**<0.001**
		Treatment	3.548	1, 12	0.084
		Age x Treatment*	**7.417**	**1, 12**	**0.018**
Figure 4b	Hippocampal p-AMPK/AMPK	Age	2.718	1, 8	0.138
		Treatment	0.181	1, 8	0.682
		Age x Treatment*	**8.283**	**1, 8**	**0.021**
Figure 4c	Hippocampal ATP	Age	3.298	1, 8	0.107
		Treatment	0.555	1, 8	0.478
		Age x Treatment	1.415	1, 8	0.268
Figure 5c	Number of BrdU positive cells	**Age***	**487.2**	**1, 11**	**<0.001**
		Treatment	2.744	1, 11	0.126
		Age x Treatment*	**7.564**	**1, 11**	**0.019**
Figure 5d	Number of DCX positive cells	**Age***	**711.407**	**1, 11**	**<0.001**
		Treatment*	**7.839**	**1, 11**	**0.017**
		Age x Treatment*	**5.054**	**1, 11**	**0.046**
Figure 6b	BDNF immunoreactivity in CA1	Age	0.842	1, 20	0.370
		Treatment	2.505	1, 20	0.129
		Age x Treatment	0.141	1, 20	0.711
Figure 6c	BDNF immunoreactivity in CA3	**Age***	**12.345**	**1, 19**	**0.002**
		Treatment*	**13.526**	**1, 19**	**0.002**
		Age x Treatment*	**11.726**	**1, 19**	**0.003**
Figure 6d	BDNF immunoreactivity in DG	**Age***	**6.933**	**1, 19**	**0.016**
		Treatment	3.069	1, 19	0.096
		Age x Treatment	3.261	1, 19	0.087

2x2 factorial ANOVA with factors as age (young versus old) and treatment (saline versus AICAR) was conducted. ANOVA, Analysis of Variance; AUC, Area under the curve; BrdU, 5-bromo-2'-deoxyuridine; DCX, doublecortin; CA, cornus ammonis; DG, dentate gyrus. Statistically significant results (set as $p < 0.05$) were indicated as bold and asterisks.

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ABSTRACT (IN KOREAN)

해마의 AMP-activated protein kinase의 반응성 감소
: 인지 노화의 신경대사적 원인

< 지도교수 : 김 어 수 >

연세대학교 대학원 의학과

장 수 아

최근 노인 인구가 증가함에 따라, 노후의 삶의 질과 건강 전반에 큰 영향을 끼치는 인지 노화에 대한 중요성이 커지고 있다. 이러한 인지 노화의 원인으로, 노화에 따른 신경 세포 에너지 대사의 손상이 중요한 역할을 한다고 알려져 왔다. AMP-activated protein kinase (AMPK) 는 에너지 항상성 조절에 결정적인 역할을 하는 효소이며, AMPK 의 적절한 활성은 에너지 대사 요구를 만족시키기 위해 필수적이다. 그러나 최근, 말초 조직에서 에너지 스트레스나 활성촉진물질에 대한 AMPK의 반응성이 노화에 따라 감소한다는 것이 보고되었다. 이러한 결과는 다양한 조직에서 노화에 따른 생물에너지대사의 장애를 설명하는 실마리가 될 수 있다. 따라서, 본 연구에서는 뇌 조직에서 AMPK 반응성이 감소하는지에 대한 여부와, 그러한 반응성의 변화가 인지 노화와 관계가 있는지에 대해 밝히고자 하였다. 이를 위해, 16주령의 젊은 쥐와 72주령의 고령 쥐에게 7일간 복강내로 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; AMPK 활성제) 혹은 생리식염수 (대조군) 을 투여한 후 매일 eight-arm maze 를 이용해 인지 기능을 평가하였다. AICAR는 젊은 쥐에서만

인지 기능을 향상시켰고 고령의 쥐에서는 효과가 없었으며, 이를 통해 인지 기능에 대한 AMPK 의 활성 효과가 고령 쥐에서 둔화된 것을 알 수 있었다. 또한, AICAR 투여시 급성 뇌실내 주입 실험에서 젊은 쥐에게서만 AMPK 그 자체의 활성도 변화가 있었으며, 고령의 쥐에서는 아만성, 급성 실험 모두에서 변화가 없었다. 흥미롭게도, AICAR 에 의한 AMPK 활성의 변화 방향이 해마 (감소) 와 골격근 (증가) 에서 반대였다. 게다가 해마의 AMPK 활성도는 인지 기능 점수와 역상관성을 보였다. 젊은 쥐에서 AICAR 처치로 인해 높아진 에너지가, 신경세포의 AMPK 를 탈활성화시켰을 것이라 가설을 세운 연구진은 급성 뇌실강내 AICAR 주사 후 에너지 변화 여부를 보기 위해 ATP를 측정했다. 예상대로, ATP 는 해마의 AMPK 활성도와 역상관성을 보여, AICAR에 의한 인지 향상에 ATP 가 기여했을 가능성을 보여주었다. 또한, AICAR 에 의한 신경세포 발생과 brain-derived neurotrophic factor (BDNF) 발현도 젊은 쥐에서만 증가하여, 노화에 따른 AMPK 의 반응성 감소의 영향을 확인할 수 있었다.

본 연구는, AMPK 의 반응성 저하가 ATP, BDNF 발현, 신경세포 발생의 촉진을 저해하여, 인지 노화의 신경대사적인 원인이 될 수 있음을 시사한다. 이 연구는, AMPK의 활성을 높이는 것 뿐만 아니라, 반응성을 회복하는 것이 인지 노화의 개선에 중요할 수 있음을 제시하는 데 의의가 있다고 본다.

핵심되는 말: AMPK, 인지 노화, 신경대사, 신경세포 발생, BDNF, 해마

PUBLICATION LIST

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